RESEARCH COMMUNICATION

Inhibition of protein synthesis in frog (*Xenopus laevis*) egg extracts by an antibody against phosphatidylinositol 4,5-bisphosphate

Thomas J. KEATING*[‡], Kiyoko FUKAMI[†] and Kenneth R. ROBINSON*[§]

*Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, U.S.A., and [†]Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan

The antibody kt10, which is directed against the phospholipid PtdIns(4,5) P_2 , inhibits protein synthesis when added to cytosolic extracts prepared from frog eggs. Addition of stable analogues of diacylglycerol and Ins(1,4,5) P_3 failed to rescue the inhibition of translation, suggesting that the effect of the antibody was not to

block hydrolysis of PtdIns(4,5) P_2 . Neomycin, which also binds PtdIns(4,5) P_2 , produced a similar reduction in protein-synthesis levels in the extract system, supporting the idea that it is the interaction of the antibody with PtdIns(4,5) P_2 that is producing the effect.

INTRODUCTION

PtdIns(4,5) P_2 is involved in a variety of signalling processes, serving as precursor for the second messengers diacylglycerol (DAG), Ins(1,4,5) P_3 , and PtdIns(3,4,5) P_3 [1,2]. In addition, PtdIns(4,5) P_2 itself has been reported to have direct effects on cytosolic proteins, serving as a membrane anchor for proteins containing pleckstrin homology domains [3] or as a cofactor for several actin-associated proteins [4]. In the latter case, PtdIns(4,5) P_2 is found in lipid–protein complexes that are outside of the membrane environment [5]. There is also growing evidence to support the idea that there is a separate phosphoinositol signalling system in the nucleus [6]. Again, the phosphoinositides appear to be directly associated with nuclear proteins rather than in the lipid bilayer [6]. Taken together, these results indicate that changes in the levels of PtdIns(4,5) P_2 could have a profound impact on a wide array of cellular functions.

To learn about second-messenger signalling in the cell cycle, we added an antibody that is specific for PtdIns(4,5) P_2 [7] to cycling extracts prepared from frog (*Xenopus laevis*) eggs [8]. Such extracts faithfully reproduce many processes of intact cells, including DNA replication [9], protein synthesis and degradation [10] and calcium homeostasis [11]. To our surprise we found that the anti-PtdIns(4,5) P_2 antibody caused a nearly complete inhibition of protein synthesis in this system. Attempts to rescue the antibody-induced inhibition by addition of hydrolysis-resistant analogues of Ins(1,4,5) P_3 and DAG were unsuccessful, indicating that the effects of the antibody were not a result of inhibiting the generation of these second messengers. Another PtdIns(4,5) P_2 -binding molecule, neomycin, also inhibits protein synthesis in this system, supporting the idea that the effect of the antibody is due to its interaction with PtdIns(4,5) P_2 .

MATERIALS AND METHODS

Reagents and solutions

[³⁵S]Methionine (15 mCi/ml) was obtained from Amersham (Arlington Heights, IL, U.S.A.); 3-deoxy-3-fluoro-D-myo-inosi-

tol 1,4,5-trisphosphate [3-F-Ins(1,4,5) P_3] from Calbiochem (San Diego, CA, U.S.A.); phorbol 12-myristate 13-acetate (PMA) and neomycin sulphate from Sigma (St. Louis, MO, U.S.A.); and *N*-phenyl octyl ether (NPOE), ETH 129 (*N,N,N',N'*-tetra-cyclohexyl-3-oxapentanediamide), tetrahydrofuran (THF), poly(vinyl chloride) (PVC), and sodium tetrakis chlorophenyl borate (NaTCPB) from Fluka (Ronkonkoma, NY, U.S.A.).

Antibody kt10, which is a monoclonal IgG_{2b} purified from ascites fluid with Protein A–Sepharose [7], was initially reconstituted at 0.14 mg/ml in PBS and was subsequently concentrated 5–20-fold with Microcon 30 kDa microconcentrators (Amicon, Beverly, MA, U.S.A.). Non-specific mouse IgG (Sigma) was prepared in the same manner for control experiments.

Preparation of frog egg extracts

Cytosolic extracts were made from eggs of *Xenopus laevis* as previously described [12], with some modifications. Eggs were expressed from ovulating females, ‘dejellied’ in 2% cysteine, and washed. The dejellied eggs were activated, washed and loaded into microcentrifuge tubes. At 10 min after activation, excess solution was removed and a layer of silicone oil was added to the tubes. The eggs were packed together by low-speed centrifugation and excess solution and oil were discarded. The tubes of eggs were left on ice for 10 min before fractionation.

The eggs were crushed and fractionated by centrifugation (10000 *g* at 4 °C for 55 min). The cytosolic fraction was collected by side puncture of the tube, and to it was added an ATP-regenerating system, proteinase inhibitors and cytochalasin B [12]. Extracts were centrifuged a second time (3 min at 12000 *g* at room temperature) to remove contaminating yolk platelets and pigment granules and again collected by side puncture. At this point the concentration of sucrose in the extract was raised to at least 0.2 M by the addition of 0.1 vol. of 2 M sucrose and the extract was divided into portions, frozen in liquid nitrogen and stored at –80 °C.

Protein-synthesis activity in *Xenopus* embryos and egg extracts is reduced substantially during M-phase [10]. Therefore, protein

Abbreviations used: DAG, diacylglycerol; 3-F-Ins(1,4,5) P_3 , 3-deoxy-3-fluoro-D-myo-inositol 1,4,5-trisphosphate; PMA, phorbol 12-myristate 13-acetate; NPOE, *N*-phenyl octyl ether; THF, tetrahydrofuran; PVC, poly(vinyl chloride); NaTCPB, sodium tetrakis chlorophenyl borate; β -ME, β -mercaptoethanol; RER, rough endoplasmic reticulum; [Ca²⁺]_i, intracellular calcium concentration.

[‡] Present address: Laboratory of Molecular Biology, University of Wisconsin, Madison, WI 53706, U.S.A.

[§] To whom correspondence should be addressed.

synthesis assays were only performed on extracts that remained in interphase for at least 30 min after being placed at room temperature.

In some cases β -mercaptoethanol (β -ME) was added to the microcentrifuge tubes at a final concentration of 0.01 % (v/v) before addition of eggs. Since most of the extracellular solution is removed from the tube when the eggs are packed in the presence of silicone oil, additional β -ME was added on top of the packed eggs, at a final concentration of 0.01 % of the volume of packed eggs.

For autoradiography, [35 S]methionine was added to egg extracts at a final concentration of 0.5 μ Ci/ μ l. Samples were taken every 10 min, combined with SDS sample buffer and boiled for 10 min. The samples were separated on a 10 % polyacrylamide gel, stained with Coomassie Blue, dried and exposed to X-ray film.

In vitro translation and trichloroacetic acid precipitation

Extracts were labelled with [35 S]methionine (Amersham, Arlington Heights, IL, U.S.A.) at a final concentration of 0.16–0.47 μ Ci/ μ l. After 30 min incubation at room temperature, samples were taken from labelled extracts and blotted on to glass microfibre discs for subsequent analysis. Trichloroacetic acid precipitation of proteins on to filter disks was performed as described previously [13]. The disks were bathed in ice-cold 10 % (w/v) trichloroacetic acid (containing 10 mM unlabelled L-methionine) for 30 min, washed three times with 10 % trichloroacetic acid for 5 min, rinsed once with 100 % ethanol and then washed for 5 min in 100 % ethanol and allowed to dry. The discs subsequently were counted in a Beckman LS-230 liquid-scintillation counter. Background levels of radioactivity were determined by treating samples of extract with 0.45 mg/ml cycloheximide, incubating these for 30 min at room temperature, and measuring levels of radioactivity in the samples using the trichloroacetic acid precipitation method described above. Background levels of radioactivity measured in this way were on average 12 % of the total amount of incorporation in PBS-treated control samples.

The amount of [35 S]methionine incorporated into each extract was normalized to the total amount of label present, which was determined by blotting dilutions of extract on to discs and counting these without further processing. The values from cycloheximide-treated extracts were subtracted from each sample to account for background levels of [35 S]methionine adhering to the filter discs. The percentage inhibition of protein synthesis was calculated according to the following formula:

$$\text{Percentage inhibition} = [1 - (x/\text{control})] \times 100 \%$$

where x is the amount of radioactivity in the test sample, control is the amount of radioactivity in a PBS-treated extract, and x and control have been normalized and background-subtracted.

Measurement of calcium with ion-specific microelectrodes

Calcium microelectrodes were prepared using a modification of a previously described technique [14]. Micropipettes with $\sim 5 \mu$ m openings were pulled from thin-walled capillary glass without internal filaments. Silanized micropipettes were backfilled with pCa 6.0 solution, after which the tip was dipped in solution 1 (0.5 ml of NPOE plus 5.5 mg of NaTCPB and 5.0 mg of ETH 129) and then in solution 2 (0.2 ml of solution 1 was added to 0.4 ml of THF in which 36.0 mg of PVC had been dissolved). The calcium microelectrodes, which had tip resistances of 2–6 G Ω , were calibrated before and after each set of measurements using pCa 7.0, 6.5, and 6.0 solutions. Measurements of [Ca^{2+}]_i were

made by dipping the calcium microelectrode and a reference microelectrode in 10–15 μ l of egg extract that was held in a small conical hole in a Delrin block. The voltage difference between the two electrodes was measured by a high-input impedance differential amplifier (model FD-223; World Precision Instruments, Sarasota, FL, U.S.A.).

RESULTS

The monoclonal antibody kt10, which is specific for PtdIns(4,5) P_2 [7], inhibited protein synthesis when added to cytosolic extracts prepared from frog eggs (Figure 1). This effect was dose-dependent, with 85 μ g/ml antibody inhibiting 95 % of the cycloheximide-sensitive [35 S]methionine incorporation (Figure 1). Non-specific mouse IgGs had no effect on protein synthesis when added at 42 μ g/ml (results not shown), the concentration at which the effect of the anti-PtdIns(4,5) P_2 IgG was nearly maximal (Figure 1). This inhibitory effect was seen in all extracts tested (22 experiments performed with 12 different extract preparations).

To determine whether the antibody-induced inhibition of translation was general or specific to a certain subset of proteins, samples of [35 S]methionine-labelled extract were separated by SDS/PAGE and autoradiographed (Figure 2). Although the overall intensity of the autoradiograph of samples from anti-PtdIns(4,5) P_2 -treated extracts was lower than those of the PBS-treated samples, the profile of labelled bands was equivalent in both cases (Figure 2). There was no change in the pattern of incorporation seen in samples taken from anti-PtdIns(4,5) P_2 antibody-treated extracts at 10 min intervals over a 90 min period (results not shown). Similar results were obtained with samples from a PBS-treated extract, with the exception that the intensity of the bands corresponding to cyclins was reduced substantially upon exit from mitosis (results not shown; [8]). Coomassie Blue

Figure 1 Concentration-dependent inhibition of protein synthesis of anti-PtdIns(4,5) P_2 antibody in frog egg extracts

Dilutions of antibody were added to aliquots of extract containing [35 S]methionine and incubated at room temperature for 30 min, after which triplicate samples were taken from each aliquot. Percentage inhibition was determined relative to control samples, where the level of radioactive label incorporated into PBS-treated extracts was taken to be 0 % and cycloheximide-treated extracts 100 %. The data presented here were obtained from experiments performed with samples from a single preparation of extract, in order to control for slight variability in the activity of the antibody from one clutch of eggs to another. Nonetheless, inhibition of protein synthesis by the antibody was seen in every extract tested.

Figure 2 Coomassie Blue (CB)-stained proteins, separated on a 10% polyacrylamide gel, and corresponding autoradiographs (ARG) of samples from extracts treated with PBS or anti-PtdIns(4,5) P_2 antibody (70 μ g/ml)

[35 S]Methionine was added at a final concentration of 0.5 μ Ci/ μ l, and 1 μ l samples were taken from the extracts at 10 min intervals and solubilized in 9 μ l of SDS sample buffer. The Coomassie Blue-stained gels and autoradiographs of the samples treated with PBS or anti-PtdIns(4,5) P_2 had similar profiles of labelled protein bands, indicating that the inhibition of translation induced by the antibody was a general effect and not specific to a particular subset of proteins. The autoradiograph from the anti-PtdIns(4,5) P_2 -treated sample has been printed darker than it appeared on the film to allow comparison of the profiles of labelled bands. Molecular-mass markers (kDa) are shown in the centre.

staining of the samples was equivalent in both cases (Figure 2), indicating that equal amounts of protein were loaded. The autoradiograph from the anti-PtdIns(4,5) P_2 -treated sample (Figure 2) has been printed darker than it appeared on the X-ray film to facilitate comparison of labelled bands between the two samples.

It is possible that, during fractionation of the frog eggs, the fatty acids of PtdIns(4,5) P_2 could become oxidized and form covalent bonds with nearby proteins [15]. If this happened to a protein that is involved in translation, then the effect of the antibody might be the result of its binding to and inhibiting such a lipoprotein created during extract preparation. As a control, therefore, extracts from the same batch of eggs were prepared in the absence or presence of 0.01 % β -ME, a potent antioxidant. In these two sets of samples, the anti-PtdIns(4,5) P_2 antibody was equally effective at inhibiting translation, producing 86 % inhibition in the β -ME-treated extracts compared with 69 % inhibition in the extracts prepared without β -ME. In addition, no differences were seen in the absolute rates of [35 S]methionine incorporation between these two groups of samples.

As the antibody kt10 has been shown to inhibit hydrolysis of PtdIns(4,5) P_2 [7] and subsequent production of Ins(1,4,5) P_3 , we investigated whether the inhibitory effects of the antibody could be due to a reduction of resting intracellular calcium concentration ($[Ca^{2+}]_i$) in the extracts. Calcium levels were measured with Ca^{2+} -specific microelectrodes in aliquots of extract that had been treated with either PBS or kt10 antibody. The resting $[Ca^{2+}]_i$ in PBS-treated extracts was 250 nM, and there was no change in $[Ca^{2+}]_i$ in the presence of 90 μ g/ml kt10, even after 30 min of incubation.

Another compound that is known to bind to PtdIns(4,5) P_2 , neomycin, was tested for its effect on translation in frog egg extracts. Neomycin inhibited protein synthesis in a dose-dependent manner, the maximal effect occurring at 1 mM (Figure

Figure 3 Concentration-dependent inhibition of protein synthesis in frog egg extracts by neomycin

Aliquots of frog egg extract were treated with neomycin and handled as described in Figure 1. The graph is a compilation of data from four separate experiments that were performed using three extract preparations. Each point represents a single determination, except for [neomycin] = 1 mM, which is the average result from two experiments that produced 100 and 103 % inhibition.

Table 1 Attempted rescue of anti-PtdIns(4,5) P_2 inhibition of protein synthesis with stable analogues of Ins(1,4,5) P_3 and DAG

3-F-Ins(1,4,5) P_3 , an hydrolysis-resistant analogue of Ins(1,4,5) P_3 , was added to extracts at a final concentration of 10 μ M, and MA, a stable analogue of DAG, was added at a final concentration of 3 μ M. The extracts were incubated for 30 min at room temperature and [35 S]methionine incorporation was assessed as described in Figure 1. The levels of antibody-induced inhibition of protein synthesis in drug-treated samples are compared directly with levels in control samples from the same experiments. The Table summarizes experiments performed with two different concentrations of antibody, 118 μ g/ml and 44 μ g/ml. Data are means $\pm$ S.D., with n as indicated in parentheses.

Addition	Inhibition of protein synthesis (%)		
	3-F-Ins(1,4,5) P_3	PMA	3-F-Ins(1,4,5) P_3 + PMA
Anti-PtdIns(4,5) P_2	90 $\pm$ 8 (3)	91 $\pm$ 11 (2)	91 $\pm$ 11 (2)
Drug(s)	78 $\pm$ 20 (3)	75 $\pm$ 0 (2)	81 $\pm$ 4 (2)
+ anti-PtdIns(4,5) P_2			

3). These data support the notion that it is the binding of the antibody to PtdIns(4,5) P_2 , rather than to some other component of the extracts, that is causing the inhibition of protein synthesis as an unrelated PtdIns(4,5) P_2 -binding molecule has the same effect.

To determine whether the products of PtdIns(4,5) P_2 hydrolysis, Ins(1,4,5) P_3 and DAG, are required for protein synthesis, we added stable analogues of these second messengers to extracts that had been treated with anti-PtdIns(4,5) P_2 antibody to try to reverse the inhibition of translation. The hydrolysis-resistant Ins(1,4,5) P_3 analogue 3-F-Ins(1,4,5) P_3 , caused prolonged increases in $[Ca^{2+}]_i$ at concentrations of 100–500 nM in *Xenopus* oocytes [16]. However, addition of 10 μ M 3-F-Ins(1,4,5) P_3 to antibody-treated egg extracts produced only a small, insignificant reduction of inhibition (Table 1). Calcium levels in an extract containing 10 μ M 3-F-Ins(1,4,5) P_3 rose to 630 nM in 10 min,

whereas $[Ca^{2+}]_i$ in a PBS-treated sample at the same time was 320 nM, indicating that 3-F-Ins(1,4,5) P_3 was effective at releasing Ca^{2+} in these extracts.

PMA is a stable analogue of DAG that produces a long-lasting stimulation of protein kinase C. When added to antibody-inhibited egg extracts, 3 μ M PMA had only a minor effect on the inhibition of translation compared with PBS-treated controls (Table 1). The effect of both drugs together [10 μ M 3-F-Ins(1,4,5) P_3 plus 3 μ M PMA] was similar to that of either one alone (Table 1), with no significant reduction in translation inhibition seen.

DISCUSSION

The results presented here describe the novel finding that an antibody directed against PtdIns(4,5) P_2 is a potent inhibitor of protein synthesis when added to cytosolic extracts prepared from *Xenopus* eggs. The inhibition of translation seems to be general to all proteins rather than a particular subset, as revealed by autoradiography. Using frog egg extracts, Kanki and Newport [10] have shown that levels of protein synthesis in the cytosol and on the rough endoplasmic reticulum (RER) are approximately equal. If the anti-PtdIns(4,5) P_2 antibody were selectively inhibiting translation in either the cytosol or the RER, then complete inhibition would be at most $\sim 50\%$. Since the antibody produced nearly complete inhibition of translation, its effect must therefore have been more general.

Attempts to reverse the effects of the anti-PtdIns(4,5) P_2 antibody with high concentrations of stable analogues of Ins(1,4,5) P_3 and DAG were unsuccessful, suggesting that the inhibitory effects of the antibody are not a result of the lack of these second messengers. Nonetheless, it is possible that global application of these compounds, even at high levels, may fail to mimic the localized production of these second messengers that occurs under more normal conditions.

The one other use of the antibody kt10 in *Xenopus* eggs was in a study of the role of calcium in cell division [17]. It was found that kt10 slowed the rate of cleavage when injected into *Xenopus* embryos at final concentrations of 33–93 μ g/ml [17], which is similar to the levels used in the experiments reported here. In light of the present results, it seems possible that this inhibition of cleavage might have been a result of reduced levels of protein synthesis instead of an interference with calcium signalling, although further experiments will be required to distinguish between these possibilities.

We found that the antibiotic neomycin, an unrelated PtdIns(4,5) P_2 -binding molecule [18,19], also produced an inhibition of translation in frog egg extracts. One caveat with the use of neomycin is the fact that it also binds to PtdIns and PtdIns4P [18], although with lower affinity than for PtdIns(4,5) P_2 . However, when coupled with the antibody-induced inhibition,

the results obtained with neomycin support the idea that it is the binding of the antibody to PtdIns(4,5) P_2 that is responsible for the observed inhibition of translation. Nonetheless, several reports have indicated that neomycin does not inhibit protein synthesis in eukaryotic cells under conditions in which it apparently binds PtdIns(4,5) P_2 [18,19]. Factors that are unique to the extract system, for example the lack of a plasma membrane, may make the translation machinery more susceptible to neomycin compared with intact cells. To investigate one possible source of 'artificial' results, we prepared egg extracts in the presence of β -ME to prevent oxidative breakdown of PtdIns(4,5) P_2 and its subsequent non-specific covalent bonding to other molecules. However, extracts prepared with and without β -ME were equally sensitive to the antibody-induced inhibition of translation.

As discussed in the Introduction, there is reason to believe that PtdIns(4,5) P_2 binds specifically to non-membrane proteins and that the interactions are important for the functions of these proteins [4,5]. Therefore a possible explanation for our results would be that the antibody kt10 binds to and inactivates a protein-PtdIns(4,5) P_2 complex that is required for translation. If this is true, it should be possible to use this antibody to purify the protein, which in turn should give new insights into the mechanisms of translation.

T.J.K. was supported by a Purdue Research Foundation Graduate Fellowship as well as the 1995 Merck Graduate Fellowship in the Department of Biological Sciences at Purdue University. Thanks are also due to Dr. Joann Otto, Dr. Allan Bieber, Dr. David Asai and Dr. Joe Venable for equipment, supplies and advice.

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